A Novel Oral Edaravone Formulation Partially Alleviates Tau Pathology and Motor Deficits in an Animal Model of Frontotemporal Dementia

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Research Article

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Abstract

Oxidative stress (OS) is a key factor in the pathogenesis of several neurodegenerative disorders and is involved in the accumulation of amyloid beta plaques and Tau inclusions. Edaravone (EDR) is a free radical scavenger that is approved for motor neuron disease and acute ischemic stroke. EDR alleviates pathologies and cognitive impairment of AD via targeting multiple key pathways in transgenic mice. Herein, we aimed to study the effect of EDR on Tau pathology in an animal model (P301L mice) of frontotemporal dementia (FTD) at two age time points representing the early and late stages of the disease. A novel EDR formulation was utilized in the study and the drug was delivered orally in drinking water for 3 months. Then, behavioral tests were conducted followed by animal sacrifice and brain dissection. Treatment with EDR improved the cognitive deficits as evaluated in Morris water maze, novel object recognition and significantly alleviated motor deficits in these mice. EDR also reduced the levels of 4-hydroxy-2-nonenal (4-HNE) and 3-nitrotyrosine (3-NT) adducts. In addition, immunohistochemistry showed that EDR reduced tau phosphorylation and neuroinflammation and partially rescued neurons against oxidative neurotoxicity. Moreover, EDR attenuated downstream pathologies involved in Tau hyperphosphorylation. These results suggest that EDR may be a potential therapeutic agent for the treatment of FTD.

1. Introduction

Tauopathies is a group of progressive neurological disorders that are characterized by intracellular accumulation of abnormal Tau inclusions in the brain and spinal cord [1]. Alzheimer's disease (AD) and frontotemporal dementia (FTD-tau) are the most common forms and account for majority of dementia cases among elderly people. Tauopathies also comprise other disorders including progressive supranuclear palsy, corticobasal degeneration, and Pick's disease [2]. The exact etiology of the disease is still unknown, but the majority of cases are sporadic [2]. Yet no disease-modifying therapy has been approved for treatment of tauopathies or AD. Therefore, understanding the underlying pathophysiology of these diseases and introducing a multitargeted therapeutic intervention is required.

The main hallmark of tauopathies is the intracellular accumulation of microtubule-associated protein Tau (MAPT) in certain brain regions involved in cognitive, behavior and motor functions [2]. In pathologic states, Tau undergoes aberrant hyperphosphorylation and self-aggregation to form neurotoxic oligomers and neurofibrillary tangles (NFT) which lead to dysfunction of axonal transport [3]. The driving force that stimulates misfolded Tau formation is multifactorial. Mutations in Tau gene [4], amyloid beta (Aβ) [5], brain injury [6], impaired insulin signaling [7] and chronic inflammation [8] are main risk factors.

Oxidative stress (OS) has been identified to be one of the cellular mechanisms that link pathogenic Tau to consequent neuron dysfunction and death [9–11]. It is defined as a state of imbalance between levels of oxidative stressors (reactive oxygen and nitrogen species; ROS and RNS) and antioxidant defense mechanisms in favor of oxidants [12] that are deleterious to cells and lead to apoptosis [13]. Multiple lines of evidence indicate that OS is a contributing factor in the neuropathology of ageing [14] and

several neurodegenerative disorders [15–17]. In AD, the presence of OS is evident by accumulation of lipid peroxidation products, increased protein oxidation and mitochondrial dysfunction [18–20]. For instance, studies showed that OS and Tau hyperphosphorylation are strongly linked together in a vicious cycle that eventually leads to cell death [21]. OS could induce Tau pathology by upregulating key enzymes involved in tau phosphorylation [22, 23]. In a feedback mechanism, misfolded Tau could further exacerbate OS via microglial activation and mitochondria dysfunction [24, 25]. Therefore, therapeutic strategies being able to target OS could possibly provide better results than those directed towards the protein deposition. Thus, the use of antioxidants such as curcumin, coenzyme Q10 (CoQ10) and vitamins as vitamin E and C has been suggested and provided promising results in animal models of dementia in terms of reducing Tau and Aβ accumulation and alleviating behavioral deficits [26–28]. However, further studies are ongoing to support their clinical benefits and effectiveness in dementia [29].

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a potent free radical scavenger, developed by Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). Currently, it is approved for treatment of acute ischemic stroke in Japan and other Asian countries and amyotrophic lateral sclerosis (ALS) by FDA. Clinical studies showed that EDR reduces neuronal damage following brain ischemia [30, 31] and inhibits motor functional deterioration in ALS patients through its neuroprotective mechanisms [32]. EDR is considered the first disease modifying therapy to be approved and could hold great promise to alleviate several other neurodegenerative diseases. Recently, treatment of APP/PS1 AD mice with Edaravone injection reduced amyloid plaque pathology and ameliorated cognitive deficits [33]. In our previous studies, we have shown that the EDR novel oral formulation can dose-dependently reverse the cognitive deficits and mood disorders of very old APP/PS1 mice (17 months) [34]. Therefore, in this study, we aimed to investigate the effect of the novel Edaravone formulation on tau pathology of P301L mice, a model of frontotemporal dementia linked to parkinsonism-17 (FTDP-17). This animal model develops age dependent hyperphosphorylated Tau in the cortex, hippocampus, and amygdala, but NFT mainly confined to amygdala, and shows behavioral and motor deficits similar to that observed in certain tauopathies in humans [35–39]. This model also shows signs of mitochondrial dysfunction that becomes significant at advanced age [40]. The novel oral formulation of Edaravone was designed earlier in our laboratory by using self-nano micellizing solid dispersion strategy and proved to be safe and effective *in vitro* and in AD animal model [41, 34].

2. Materials And Methods

2.1. Materials

The novel Edaravone formulation was made by WuxiApptec and obtained from Suzhou Auzone Biotech, China.

The vehicle control Soluplus was provided as a gift from BASF Australia Ltd. Primary antibodies used in this study are listed in **Table 1** in supplement material. Biotinylated IgG conjugated secondary antibodies for immunohistochemistry were obtained from Thermo Fisher Scientific, Australia. IRDye® 680RD and 800CW conjugated secondary antibodies for western blotting were obtained from LI-COR Millennium

Science, Victoria, Australia. Protease and phosphatase cocktail inhibitors were purchased from Thermo Fisher Scientific, Australia (Roche Diagnostics GmbH, Mannheim, Germany). VECTASTAIN ABC kit was purchased from Vector laboratories, USA. Pierce™ BCA Protein Assay Kit was obtained from Thermo Fisher Scientific, Australia. Other chemicals were purchased from Sigma Aldrich, Australia.

2.2. Animals

The breeders of male and female P301L mutant mouse (pR5-183) mouse, bred on C57BL/6 X DBA/2 F2genetic background, expressing the longest human MAPT brain isoform (Tau40) under the control of the murine Thy1.2 promoter and with parkinsonism linked to chromosome 17 [37], were obtained from Götz laboratory in Queensland Brain Institute and bred in the Core Animal Facility, University of South Australia. C57BL/6 (WT) mice were used as controls in behavioral experiments. Animals were kept under standard conditions of 22°C room temperature and a 12 h light/dark cycle with food and water provided ad libitum. All procedures were approved by the Animal Ethics Committee of the University of South Australia (U17/14 and U13/18) and compliant with the South Australian Animal Welfare Act and the "Australian code of practice and use of animals for scientific purposes" [42].

2.3. Treatment Protocol

In order to evaluate the influence of oral EDR treatment on Tau pathology in P301L mice as the pathology progresses, we included two groups of animals **A** and **B**, one at the age of 9–10 months and an older cohort with an age of 21 months, respectively. **Group A**: n = 32, 18 P301L (11 males and 7 females) and 14 WT mice (4 males and 10 females), and **Group B**: n = 12, 8 P301L (4 males and 4 females) and 4 WT male mice. WT mice were used as controls for behavioral tests and have not received any treatments. P301L mice in both age categories were divided equally into two groups: a control group that was given the vehicle of drug (Soluplus [43]) and a treatment group that received oral Edaravone formulation dissolved in drinking water at a dose of 24 mg/kg/day for 3 months as follows: **Group A**: Vehicle treated: n = 9 (3 females and 6 males) and EDR treated: n = 9 (4 females and 5 males), **Group B**: vehicle treated: n = 4 (2 males and 2 females), EDR treated: n = 4 (2 males and 2 females). Animals were then subjected to behavior and motor tests that lasted for 3 weeks while still receiving treatment. After that, animals were humanely killed by inhalation of overdose of CO_2 and transcardially perfused with ice cold saline. Brain tissues were harvested from each mouse and divided into two halves. The left hemisphere was snap frozen in liquid nitrogen and then stored at -80°C for subsequent biochemical analysis while the right hemisphere was fixed in 4% paraformaldehyde in PBS for histological analysis. Schematic illustration of the study is shown in scheme 1.

2.4. Behavioral Tests

2.4.1. Morris Water Maze (MWM)

MWM was used to assess spatial reference learning and long-term memory [44]. The test was conducted in a black circular pool filled with water at 21 ± 2°C that was made opaque using non-toxic white powder paint. The arena was divided into 4 quadrant zones using the ANY-maze software (Stoelting Co., USA). A

white circular platform was placed in one of the four quadrants. The test was done over 6 consecutive days. On the 1st day, each animal was trained to find the visible platform within 60 seconds with the help of visual cues placed around the pool walls. In the next four days, the platform was submerged under water for 1-1.5 cm, and time required to find the hidden platform (escape latency) was calculated. Each animal was subjected to 4 trials/day (30–60 min interval), in each trial, the animal was placed at different starting point facing the pool side walls. If the animal failed to locate the platform, it was gently directed and allowed to sit on it for 30 seconds before being returned to the cage. In the last day, the platform was removed, and each animal was exposed to one probe trial for 60 seconds. Time spent in the target quadrant zone in which the platform was placed as well as number of platform site crossing were recorded using ANY-maze video tracking system v4.5. Escape latency during the hidden platform trials, swim speed and distance were also recorded.

2.4.2. Open field test and elevated plus maze test

To assess anxiety and exploratory behaviors, the open field and elevated plus maze tests were conducted as described previously [35]. In the open field test, each mouse was placed individually at the border of a square PVC arena (50 x 50 x 40 cm), and allowed to explore the arena freely for 5 min. The arena is divided into inner zone (central) and outer zone with the software tracking system. Anxiety and exploratory behaviors were assessed by measuring the number of rearing episodes, time immobile and time spent in central zone. Locomotor activity was assessed by measuring total distance travelled and number of zone transitions.

The elevated plus maze was used to assess amygdala related anxiety behavior. It is a runway consisted of 4 opposing arms each with 46 cm length and 5 cm width elevated to a height of 70 cm above the floor. Two opposing arms are open and the other two are closed. Each animal is placed at the end of a closed arm facing the wall and allowed to explore the maze freely for 5 min. The following parameters were measured: total distance travelled, number of zone transitions, total number of head dips, percentage of time spent in open arms and percentage of open arm entries to total arm entries.

2.4.3. Novel Object Recognition Test (NORT)

This test was conducted to evaluate recognition memory and preference to explore novel objects [45]. Following habituation to the test arena, the animal was exposed to two similar objects placed symmetrically away from the center for a duration of 5 min (sample phase). 2–3 hours later, one of the objects is removed and replaced by a different one (novel) and then each animal was allowed to explore the two different objects for 5 min (test phase). Time spent exploring both objects was recorded. Recognition index (RI) was calculated by dividing the novel object exploration time by the total time spent to explore both objects and multiplied by 100.

2.4.4. Y maze spontaneous alternation and Y maze novel arm

These tests were used to evaluate working and short-term memory as described previously [45]. The test was carried out in a Y-shaped arena with 3 opaque arms placed at a 120° angle from each other. In Y-maze novel arm test, each animal can explore two open arms freely for 5 min while the third one is blocked. The blocked arm is then opened, and the animal again freely explores all the three arms for 5 min (test phase). Tendency to explore the novel arm was assessed by measuring the time spent in the novel arm and number of novel arm entries. Y maze spontaneous alternation test was carried out 1 week later in the same arena, but the arms were rotated 45° before starting. In this test, each animal explores the maze freely with all arms open for 5 min for a single trial. Percentage of spontaneous alternation and total number of arm entries were recorded. Subjects showing a total of less than 7 arm entries were excluded. Healthy animals should display tendency to visit a less recently visited arm, thus showing higher alternation percentage.

2.4.5. Hind Limb Clasping Test

Limb clasping is a motor test to evaluate deficits in the corticospinal function. Motor function deficits have been found in Tau transgenic animal models [46] and were observed in late stages of AD and FTD patients [47, 48]. P301L mice start to show progressive hind limb retraction at 9–12 months of age. When the animal is suspended by the tail, normally it elicits escape response by spreading out the hind limbs and extending the toes. Deficits in this response is scored based on the severity from 0–4 depending on the ability to spread-out the hind limbs and extend the toes. The animal was suspended from the tail for 10 sec and the position of the hind limb is observed. If the hind limbs are steadily splayed outward away from the abdomen, it is given a score of 0. If one hindlimb is retracted and toes are normally splayed, it is given a score of 1. If both hindlimbs are partially retracted toward the abdomen, while the toes are normally splayed, it is given a score of 2. If its hindlimbs are totally retracted and touching the abdomen with curled toes and immobility, it is given a score of 3. If forelimbs and hind limbs are crossed and toes are curled and immobile, it is scored 4.

2.5. Immunohistochemical analysis (IHC)

Coronal brain sections were cut at 35 µm thickness using Leica cryostat (CM3050S, Germany) and stored in antifreeze solution (0.1M PBS containing 30% glycerol and 30% ethylene glycol) at -20°C till further immunostaining. Free floating brain sections were treated with 1% SDS in PBS (pH 7.4) for 10 min for antigen retrieval then incubated with 3% H_2O_2 in PBS for 15 min to inhibit endogenous peroxidases. After washing, sections were blocked with 20% normal horse serum (NHS) in PBS for 2 hours at room temperature (RT). Sections were incubated with the primary antibodies diluted in 1%NHS in PBS and 0.3% Triton-X 100 overnight at 4°C. Sections were then treated with biotinylated secondary antibody at RT for 2 hours followed by avidin/biotinylated horseradish peroxidase (ABC kit) and then developed with diaminobenzidine (DAB) for 5 min. Immuno-stained sections were mounted on slides and dehydrated, cleared with xylene and cover slipped for imaging and analysis. Slides were scanned with NanoZoomer S60 Automated Digital slide scanner (HAMAMATSU, Australia). Image analysis was done with Image J software (NIH, Bethesda, MD, USA). Primary antibodies and concentrations used in IHC are listed in **Table 1** supplement material.

2.6. Western Blotting analysis

Frozen hemi-brains were homogenized using liquid nitrogen and then sonicated with RIPA buffer (50mM Tris-HCl, 150mM NaCl, 2mM EDTA, 0.1% SDS, 1% Sodium deoxycholate and 1% Nonidet-40) (pH 7.4) at 4°C containing protease and phosphatase cocktail inhibitors. The homogenate was then centrifuged at 15000 rpm for 30 min and the supernatant (RIPA soluble extract) was collected. Total amount of protein was measured using BCA assay (Bicinchoninic Acid) total protein assay kit. Samples with equal protein concentrations were separated on 10–12% SDS-PAGE and electro-transferred onto 0.2 µm nitrocellulose membranes (GE Healthcare, Life science, Germany). Blots were blocked with 5% milk in Tris-HCl buffer with 0.5% Tween 20 for 1 hour at RT then probed with primary antibodies overnight at 4°C. Blots were then incubated with IRDye conjugated secondary antibodies and imaged using Odyssey® CLx Infrared fluorescent scanner. Image analysis was then done using Image Studio Lite software (Li-COR Bioscience). Primary antibodies and concentrations used in western blotting are listed in **Table 1** supplement material.

Statistical analysis

Results were represented as mean ± SEM. One-way or two-way analysis of variance (ANOVA) when appropriate followed by Tukey's Multiple Comparison Test or Fisher LSD post-hoc tests were used to analyze behavioral test results between WT, control and treated groups. Unpaired two-tailed t test was used to compare means of two groups (control and treated P301L mice). Analysis was done using GraphPad Prism v 9.0.0 (GraphPad Software). P value < 0.05 was considered statistically significant for all comparisons.

3. Results

3.1. Edaravone improved spatial learning memory of P301L mice in MWM test

To assess the effect of EDR on spatial reference learning, vehicle and EDR treated mice with their WT littermates were tested in MWM. Results showed that the average escape latency during all hidden platform trials did not differ between groups. All groups learned to locate the hidden platform as evident by decrease in latency time during the acquisition phase {14 months (n = 9–14/group): time effect: $F(1.757, 15.81) = 0.9567$; $p = 0.3944$, treatment effect: $F(2, 9) = 0.2654$; $p = 0.7727$, treatment/time effect: $F(6, 27) = 0.3892$; $p = 0.8794$, {25 months [n = 4/group]: time effect: $F(2.748, 24.74) = 1.848$; $p = 0.1681$, treatment effect: $F(2, 9) = 3.746$; $p = 0.0655$, treatment/time effect: $F(6, 27) = 1.656$; $p = 0.1705$} (Fig. 1a and 1b). However, EDR treated mice and WT littermates showed reduced average escape latency compared to vehicle controls.

In the probe trial, 14 months old control P301L mice spent less time in the target quadrant zone compared to WT ($F(2,29) = 3.821$; $p = 0.033$) and showed the least number of platform site crossing

(F(2,29) = 0.8158; p = 0.452). EDR treated mice performed better than vehicle controls as there was no difference in the time spent in the target zone between WT and EDR mice (p = 0.6891) (Fig. 1c, 1e). No difference in the time spent in the target zone was found in 25-month-old mice (F (2,9) = 0.1265, p = 0.8827). However, EDR treated mice showed significantly higher number of platform site crossing compared to vehicle control group (F (2, 9) = 7.929; p = 0.0236), and nearly equal to WT group (p = 0.943). These results suggest that EDR improved acquisition and search accuracy of P301L mice (Fig. 1d, 1f).

3.2 Edaravone altered the exploratory behavior of P301L mice in open field and elevated plus maze tests

Mice were tested in open field and elevated plus maze to evaluate the effect of Edaravone treatment on anxiety, exploratory and locomotor behavior. In open field test, control P301L mice showed significantly longer immobility time % (F (2,9) = 5.336, p = 0.0256) compared to WT littermates. They also displayed less tendency to explore the central (anxious) zone (less time in central zone) (F(2,9) = 7.576, p = 0.0139), and travelled less distance (F(2,9) = 7.307, p = 0.005), with less average speed (F(2,9) = 7.193, p = 0.0046) compared to WT mice. They also showed less tendency of exploration as evident by reduced rearing behavior (F(2,9) = 3.947, p = 0.038). EDR treatment did not improve or reverse such behavior in the 14 months old group. The results of immobility time and the time spent in the central zone were similar among younger mice and no difference was found between vehicle and EDR groups. At the old age group, both control and EDR treated P301L mice spent significantly less time in central zone compared to WT mice (F (2, 9) = 9.201; p = 0.0089 for control P301L vs WT, p = 0.0175 for EDR treated P301L vs WT). But there was no difference between vehicle and EDR treated mice (p = 0.89). Also, no significant difference was observed between P301L and WT mice for total distance, average speed, rearing episodes, number of zone transitions (line crossing) and immobility time %, possibly due to small sample size. (Fig. 2a-f).

In the elevated plus maze task, no significant difference was found between WT and P301L mice at both age groups regarding time (14 months old group: F (2, 29) = 1.742; p = 0.193, 25 months old group: F (2, 9) = 2.244; p = 0.1619) and entries to open arms (14 months old group: F(2, 29) = 2.162; p = 0.133, 25 months old group: F (2, 9) = 0.855; p = 0.457), distance (14 months old group: F(2, 29) = 0.5682; p = 0.5727, 25 months old group: F (2, 9) = 0.965; p = 0.417), and total number of head dips (14 months old group: F(2, 29) = 2.923; p = 0.0697, 25 months old group: F (2, 9) = 0.309; p = 0.742). However, we observed that control P301L mice spent more time in the open arms and showed overall higher exportation and spontaneous locomotion. In contrast, EDR treated littermates tended to spend more time in the closed arms similar to WT littermates which may indicate that they can recognize their starting location with more protected head dips. We also found that EDR significantly increased the percentage of protected head dips of P301L mice at 25 months old group compared to vehicle controls (F (2, 9) = 9.575; p = 0.011 vs controls) (Fig. 2g-k).

3.3 Edaravone did not affect the working memory in Y maze novel arm test and spontaneous alternation test

In order to investigate the effect of EDR on spatial and short-term working memory, mice were tested in the Y maze test. In the spontaneous alternation task, all mice in the 14 months old group showed the same percentage of spontaneous alternation ($F(2, 29) = 0.7303$, $p = 0.4904$). However, in the 14 months old group, WT mice showed more total arm entries compared to control ($F(2, 29) = 4.714$, $p = 0.08$) and significantly more than EDR treated mice ($p = 0.02$). Similarly, in the 25 months old age group, there was no significant difference in spontaneous alternation % among groups ($F(2, 9) = 2.928$, $p = 0.1048$), but control P301L mice showed the highest alternation (70%) while WT and EDR treated mice alternated between 50 and 55%. On the other hand, the total number of arm entries was significantly higher in WT mice compared to control P301L mice ($F(2, 9) = 4.344$, $p = 0.042$), while EDR treatment slightly increased it but the difference was not statistically significant compared to both WT ($p = 0.580$) and vehicle treated mice ($p = 0.199$) (Fig. 3a, **b**). In the Y maze novel arm task, there was no significant difference in novel arm time (14 months old group: $F(2, 29) = 0.3917$; $p = 0.679$, 25 months old group: ($F(2, 9) = 1.145$, $p = 0.361$) or entries (14 months old group: $F(2, 29) = 0.08491$; $p = 0.919$, 25 months old group: $F(2, 9) = 0.3302$, $p = 0.727$) between WT and P301L mice in both age groups (Fig. 3c, **d**).

3.4. EDR improved the recognition memory in P301L mice

Novel object recognition test was used to evaluate the effect of EDR on the animal tendency to explore novel objects over familiar ones. Results showed that there was no significant difference in the recognition index between groups in the 14 months old group ($F(2, 29) = 0.7909$, $p = 0.4630$). However, in the 25 months old group, control P301L mice showed less interaction with the novel object (RI = 35%) compared to WT mice (RI = 51%), while EDR significantly improved the recognition memory of P301L treated mice ($F(2, 9) = 5.099$, $p = 0.0269$, RI = 70%) compared to their control littermates and performed even better than WT mice ($p = 0.2429$) (Fig. 4a).

3.5. EDR attenuated motor deficits in P301L mice

In order to test the effect of EDR on the motor deficits observed in P301L mice, limb clasping test was carried out. In both age groups, control P301L mice showed significantly higher limb clasping score compared to WT (14 months old: $F (2, 29) = 15.20$, $p < 0.0001$, 25 months old: $F (2, 9) = 8.471$, $p = 0.007$) that agrees with previous findings observed in that model [49]. EDR reduced the limb clasping score compared to vehicle controls (14 months old: $p = 0.0217$, 25 months old: $p = 0.1541$) (Fig. 4b). These results suggest that EDR rescued motor function in P301L mice.

3.6. Effect of EDR on phosphorylated Tau expression in P301L mice

To study the effect of EDR treatment on Tau pathology, we did immunostaining and western blotting of whole brain homogenate for epitope pS396 Tau and AT8 (an antibody for phosphorylated Tau at

Ser202/Thr205). Given the initial characterization of P301L mice, Tau aggregates can be detected in several brain areas; early in the basolateral nucleus (BLA) of amygdala and later in the hippocampus (< 18 months old) [37, 35]. The expression of endogenous mouse tau and mutant human tau was detected by mouse tau 5 and HT7 antibodies, respectively. While neurons of WT littermates did not show any immunoreactivity towards HT7 antibody (data not shown). IHC revealed that EDR treatment significantly reduced the immunoreactivity of pS396 in the hippocampus (14 months old: $t(34) = 2.309$, $p = 0.027$; 25 months old: $t(14) = 3.681$, $p = 0.0025$) and amygdala (age = 14 months old: $t(35) = 1.692$, $p = 0.099$, age = 25 months old: $t(14) = 3.681$, $p = 0.0293$) compared to controls (Fig. 5A, 7A). In the same context, western blotting also showed modest reduction in the expression levels of pS396 and AT8 compared to total HT7, though, seemed to be less sensitive than IHC to detect any change in the total brain homogenate. The expression levels of total mouse tau and mutated human tau were not altered in control and treated mice. But, interestingly, HT7 expression level was significantly reduced in 25 months old EDR treated mice compared to their control littermates ($t(14) = 3.681$, $p < 0.0407$, **Fig. 6a and 8a**). We also performed two-way ANOVA to compare the hyperphosphorylated Tau expression levels between the two age groups. We found significant increase in levels of pS396 Tau and AT8 in the older age group compared to both vehicle and EDR treated 14 months old P301L mice, that indicated the buildup of the pathology with aging. Interestingly, EDR significantly reduced the expressions levels of pS396 phosphorylated Tau $F (3, 14) = 17.48$, $p < 0.0001$) and AT8 ($F (3, 14) = 17.12$, $p < 0.0001$) in the 25 months old P301L mice compared to the age matched vehicle controls (please refer to supplement Figs. 14,15 and 16 in supporting information).

3.7. EDR treatment altered GSK-mediated Tau phosphorylation

To assess the effect of EDR on the activity of Glycogen synthase kinase 3β (GSK-3β), we performed western blotting for the total protein level and its phosphorylated form at Ser9 (pS9-GSK3β). GSK-3β is a serine/threonine kinase and involved in aberrant Tau phosphorylation in neurodegenerative diseases [50, 51]. Western blot revealed that P301L mice showed significantly reduced levels of the inactive pS9-GSK3β (inhibited) form to the total protein compared to age matched WT mice in the 14 months old group, but no difference among all groups in the older age (14 months old: $F (2, 23) = 14.38$, $p < 0.0001$; 25 months old: $F (2, 8) = 0.1093$, $p = 0.8978$). However, total GSK-3β protein levels did not change. On the hand, EDR did not increase the levels of the phosphorylated form of GSK-3β in treated P301L mice compared to vehicle controls. This might indicate that the effect of EDR treatment on Tau hyperphosphorylation is mediated through different mechanisms but not mainly through altering the activity of GSK-3β (Fig. 6b and 8b). Performing Two-way ANOVA for samples run on same gel to compare the expression levels of pS9-GSK3β among groups of different ages, we found significant increase in the levels of the phosphorylated form to the total protein levels in the EDR treated older P301L mice compared to age matched vehicle controls and to both vehicle and EDR treated younger P301L mice ($F (3, 14) = 14.74$, $p = 0.0001$ please refer to supplement Fig. 19 in supporting information).

3.8. EDR treatment reduced neuroinflammation in the brains of P301L mice

In order to study effect of EDR on neuroinflammation, we examined the expression levels of glial fibrillary acidic protein (GFAP); a marker of astrocytosis and CD45; a leukocyte common antigen and Iba-1; both detect activated microglia. IHC showed that the density of GFAP-immunoreactive astrocytes was reduced in EDR treated mice from 9.4–4.7% ($t(6) = 3.760$, $p = 0.0094$) in the hippocampus and from 7.1–4.4% in the amygdala ($t(6) = 3.569$, $p = 0.0118$, **Fig. 7C**) at 25 months old, and showed a trend towards reduction in the hippocampus of 14 months old group compared to controls ($t(16) = 0.8769$, $p = 0.393$, **Fig. 5C**). In addition, EDR also significantly reduced CD45 positive microglia in the brains of old P301L mice ($t(6) = 2.576$, $p = 0.042$, **Fig. 7E**). Western blotting of the whole brain homogenate showed subtle changes in GFAP expression levels of in both age groups (Fig. 6c and 8c), and significant reduction in Iba-1 expression in 25 months age P301L group compared to age matched controls ($F(2,9) = 6.412$, $p = 0.0186$, **Fig. 8c**). These results suggest that EDR oral formulation displayed some anti-inflammatory effects, which is more obvious in the older age group as Tau pathology progresses with aging.

3.9 EDR treatment attenuates loss of synaptic plasticity and protected neurons in the brain of P301L mice

Then, we studied the effect of EDR on the expression levels of some synaptic proteins including post synaptic density-95 (PSD-95), vesicle-associated membrane protein-2 (VAMP-2) and synaptophysin. Immunoblotting results showed that EDR oral formulation significantly increased the expression of synaptophysin in the 14 months old group ($F(3, 22) = 4.947$, $p = 0.0089$ vs age matched controls, two-way ANOVA, supplement Fig. 18), while slightly improved the expression levels of other synaptic proteins (Fig. 6d and 8d). To study the effect of EDR on neuron cell viability, we performed NeuN western blotting and IHC to detect nuclei of mature neurons in aged mice when massive neuron loss is evident. No significant difference was found in NeuN immunoreactivity (calculated as % of area fraction) in all regions of the hippocampus, dentate gyrus (DG) and neocortex of EDR treated P301L mice compared to controls (**Suppl. Figure 2**), although data showed a trend towards improvement in EDR treated mice. Western blotting also showed no differences in NeuN protein expression between WT, controls and EDR treated in both age groups (Figs. 6c and 8c). These results might suggest that EDR oral formulation was able to improve synaptic function and, to some extent, inhibit neurotoxicity in aged P301L mice.

3.10. EDR alleviates oxidative stress in P301L mice

4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT) protein adducts are considered a biomarker of oxidative and nitrosative damage [52, 53]. Results showed that EDR produced small but significant reduction in the levels of 4-HNE (Fig. 6t, $t(16) = 6.728$, $p < 0.0001$, 14 months old) and 3-nitrotyrosin (3-NT) (Fig. 8t, $t(6) = 5.979$, $p = 0.001$, 25 months old) in the brains P301L mice as evident by western blotting.

4. Discussion

OS is a key component of the pathophysiology of several neurodegenerative disorders [40, 54, 33]. Accumulation of OS markers and activation of antioxidant defenses has been reported in patients and directly correlates with the age and disease progression [16, 18]. Similar findings were obtained from animal models of dementia [55]. Tau aggregate is a primary culprit in AD and tauopathies. Studies revealed that there is a strong relationship between OS and abnormal Tau aggregation [56]. *In vitro* studies showed that 4-HNE and lipid peroxide products could facilitate Tau polymerization and aggregation [57, 58] and can alter the expression of kinases in favor of Tau hyperphosphorylation [22, 59]. Also, nitro-tyrosine residues have been found colocalized with NFT in the brain of AD patients [60]. In addition, hyperphosphorylated Tau can provoke OS by altering the mitochonderial dynamics and microglial activation. This cycle results in cascade of events that eventually lead to cell death [61–63]. Therefore, it is assumed that targeting oxidative stress pathways could result in blocking multiple cascades involved in disease pathogenesis and therefore halt the disease progression.

EDR is a potent antioxidant and free radical scavenger. It can penetrate the blood brain barrier (BBB) where it acts centrally by reducing the oxidative injury in neurons through eliminating free radicals and lipid peroxidation products [64–67]. EDR also showed neuroprotective effects and suppressed proinflammatory responses following brain ischemia [68, 69]. Thus, in the present work, we aimed to investigate if EDR treatment for a 3-months period could alleviate cognitive deficits and Tau pathology in P301L mice.

To achieve our goal, we used a novel oral formulation of Edaravone that was previously designed by Parikh et al [41]. Edaravone exhibits poor oral bioavailability (5.23%) [70] that requires its administration trough intravenous route. Parikh et al. developed a novel oral formulation using self-nano-micellizing solid dispersion of EDR and Soluplus as a drug carrier in a ratio of 1:5. EDR/Soluplus formulation displayed high aqueous solubility and enhanced oral bioavailability of EDR by 10.2-, 16.1-, and 14.8-fold compared to EDR suspension at 46, 138, and 414 µmol/kg doses in rats. Authors also reported that effective micellar encapsulation of EDR by Soluplus reduced the intestinal metabolic degradation of the drug and inhibited P-glycoprotein efflux, which could explain the improved oral bioavailability of this formulation. Furthermore, this formulation was assessed *in vivo* and was found to be able to alleviate cognitive deficits of AD mice at 17 months old AD mice in a dose dependent manner [34].

Therefore, in this study, P301L mice were treated with the EDR formulation at a dose of 24 mg/kg/day dissolved in drinking water for 3 months. This approach was more acceptable than daily EDR injection or gavage in terms of animal welfare as it will not impact the animal behavior or cause any stress. P301L mice showed impaired spatial reference memory in MWM that deteriorate with aging. EDR improved the spatial learning by reducing the escape latency and significantly improved the accuracy to locate the platform in the probe trial as evident by increased number of platform site crossing in old P301L mice. This indicates that EDR preserved the cognitive function at that age. In line with these results, we found that EDR significantly increased the recognition index in NORT in old P301L mice, suggesting that EDR

rescued the working memory in old mice when it is severely impaired. However, no difference was found between animals in the 14 months old group and may indicate intact working memory at that age.

In the open field test, P301L mice showed more anxiety and less exploratory behavior, however, EDR did not reduce such anxiety. Results from EPM were not consistent with those of the open field. In that test, control P301L mice seemed to be less anxious than WT and EDR treated littermates in both age groups. They visited the open arms more frequently and displayed more unprotected head dips. These results agree with previous findings showing that P301L mice exhibited modest disinhibition of exploratory behavior that becomes more pronounced with aging [35, 71] and is accompanied by reduced anxiety levels. Such behavior suggests the presence of abnormality in amygdala-dependent tasks and most likely due to Tau accumulation in this region of the brain. Interestingly, EDR treated mice behaved more similarly to WT littermates, which might suggest that EDR could partially reverse the amygdala dysfunction.

In Y maze spontaneous alternation, all animals alternated similarly, however, P301L mice showed fewer number of total arm entries than WT littermates. EDR significantly increased the number of total arm entries in old P301L mice. Similarly, no significant difference was found between animals in the Y maze novel arm test, that agrees with previous reports indicating intact spatial working memory in younger P301L mice (at 6 and 11 months of age) [35]. In line with previous reports, P301L mice showed minor motor impairment as evident by increased limb clasping score [37]. EDR treatment reduced the limb clasping score in both age groups, an indication of improved motor function following EDR treatment. This result is consistent with human studies in amyotrophic lateral sclerosis (ALS) which show the efficacy in the improvement of motor functions [32, 72]. Our data in the present study suggest that the oral EDR formula may be more compliant to treat ALS patients than the injection formula.

EDR produced modest reduction in phosphorylation of both murine and mutant human Tau at different epitopes in CA1 of the hippocampus and BLA of the amygdala. Further immunoblotting analysis revealed slightly increased levels of pS9-GSK3β which is the inactivated form of GSK-3β, a main kinase involved in abnormal Tau phosphorylation. Therefore, it is expected that Edaravone reduced Tau phosphorylation through inhibiting GSK-3β kinase activity. However, given that no significant difference was found in the levels of pS9-GSK between control and EDR treated mice, further investigation is required to study the effect of EDR on Tau phosphorylation and its mechanisms of action.

We also found a significant reduction in oxidative and nitrosative stress as evident by reduced levels of 4-HNE and 3-NT modified proteins in the brain of EDR treated mice. Therefore, it is likely that EDR suppresses tau hyperphosphorylation by attenuation of oxidative stress and OS-induced inflammation.

Activated astrocytes and microglia accompany Tau lesions [73] and directly correlate with the extent of cognitive decline [74]. EDR treatment reduced GFAP expression in different brain regions and microgliosis in old mice as evident in IHC and western blotting. We also found that EDR improved the levels of synaptic proteins in both age groups. Our results are supported by previous findings showing that EDR injection significantly attenuates neuroinflammation and improved synaptic plasticity in AD mice [33].

The anti-inflammatory effect of EDR could result from inhibition of abnormal Tau phosphorylation with subsequent improvement in synaptic function. In addition, the number of mature neurons in the brain of 25 months old treated mice showed a trend towards increase in all regions of the hippocampus, DG, and neocortex. These results might indicate the neuroprotective effect of EDR against oxidative neurotoxicity and agree with previous studies [69]. The neuroprotective activity of EDR could be attributed to its antioxidant and indirect anti-inflammatory effects.

In summary, we reported that treatment with oral Edaravone formulation was able to improve both memory and motor functions in P301L mice and reduce tau phosphorylation. We believe that the effect of EDR was significant at advanced ages in that model possibly because the OS and mitochondrial dysfunction become significant and obvious in P301L transgene as a result of Tau pathology aggravation as previously reported by David et. Al [40]. These results clearly support the possible use of Edaravone to alleviate FTD.

However, this study has few limitations. First, the limited number of animals included in the old group. Second, only one therapeutic dose has been used although the maximal effect at the dose of 24 mg/kg/day was achieved in APP/PS1 mice in our previous study [34].

Conclusions

Herein, we highlighted the benefits of the anti-stroke drug Edaravone for the therapy of AD and other tauopathies by targeting multiple key pathways including OS, Tau phosphorylation and neuroinflammation. EDR as intravenous injection has proven to be clinically safe and effective for ALS and ischemic stroke, and our study further supports its efficacy in prevention and treatment of other neurodegenerative disorders. Besides, the oral drug administration is expected to be more convenient and safer than IV injection. Therefore, clinical trials are required to approve the efficacy of EDR and its effective doses in dementia and other oxidative stress-related diseases.

Declarations

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Conflict of interest

XFZ is one of the inventors of Chinese patent 200610149832.9. The authors report no other conflicts of interest in this work.

Contributions

Larisa Bobrovskaya and Xin-Fu Zhou contributed to the study conception and design. Material preparation, experiments, data collection and analysis were performed by Sally Kelliny. Jing Xiong collaborated in animal studies. The first draft of the manuscript was written by Sally Kelliny and all authors commented on previous versions of the manuscript. Larisa Bobrovskaya and Xin-Fu Zhou supervised the study. All the authors reviewed and approved the final version of the manuscript submitted.

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Data Availability:

All of the data that was generated and analyzed in this study are included in this article.

Compliance with Ethical Standards

Conflict of interest

Authors declare no conflict of interest.

Ethical approval

All procedures were compliant with the approved protocol (U17-14, U13-18) from Animal Ethics Committee of the University of South Australia and the South Australia animal welfare act and the “Australian code of practice and use of animals for scientific purposes”.

Consent to Participate.

Not applicable.

Consent for Publication

Not applicable.

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Figures

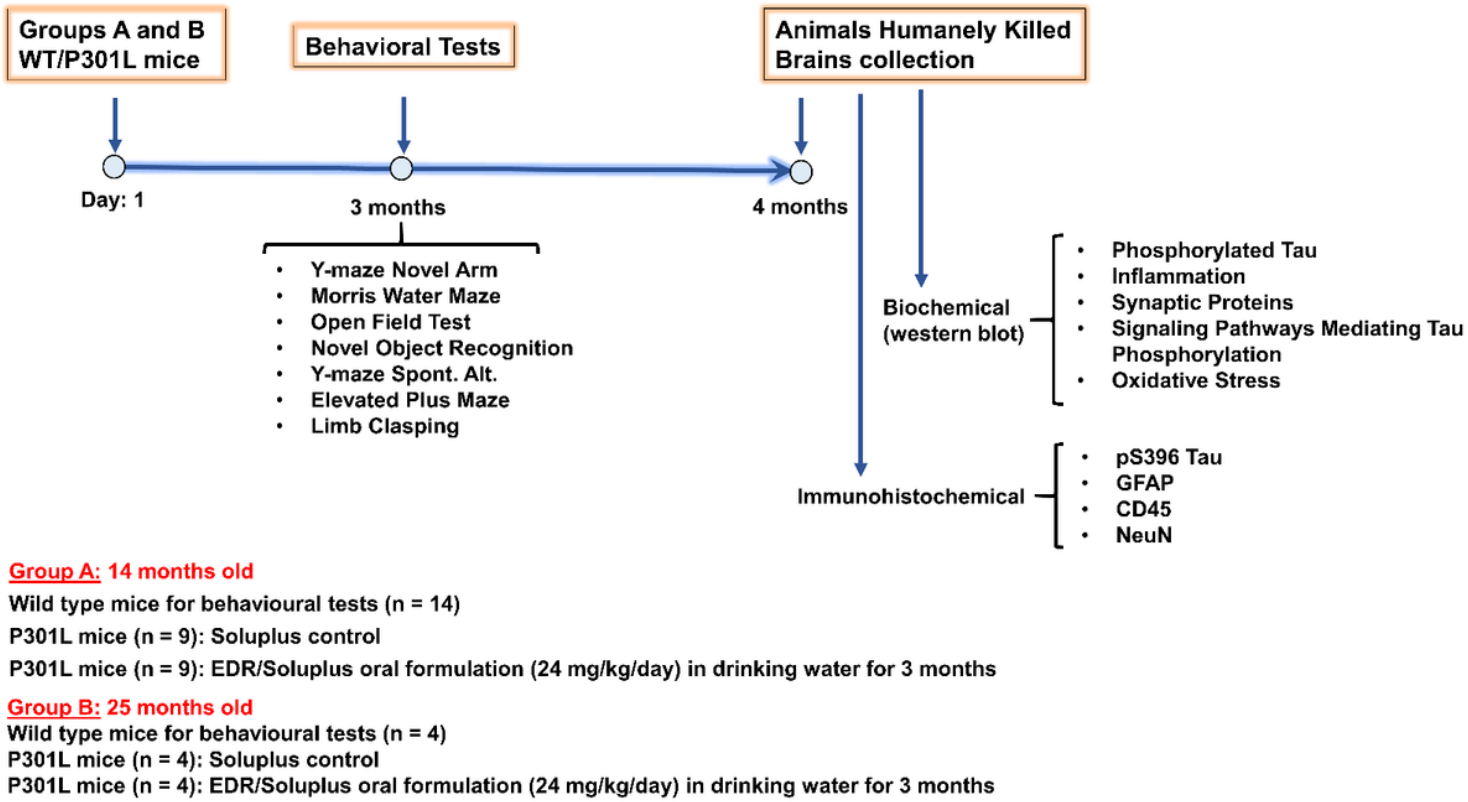

Figure 1

Schematic illustration of the study design showing animal groups, behavioral tests performed and differed biochemical and immunohistochemical analysis.

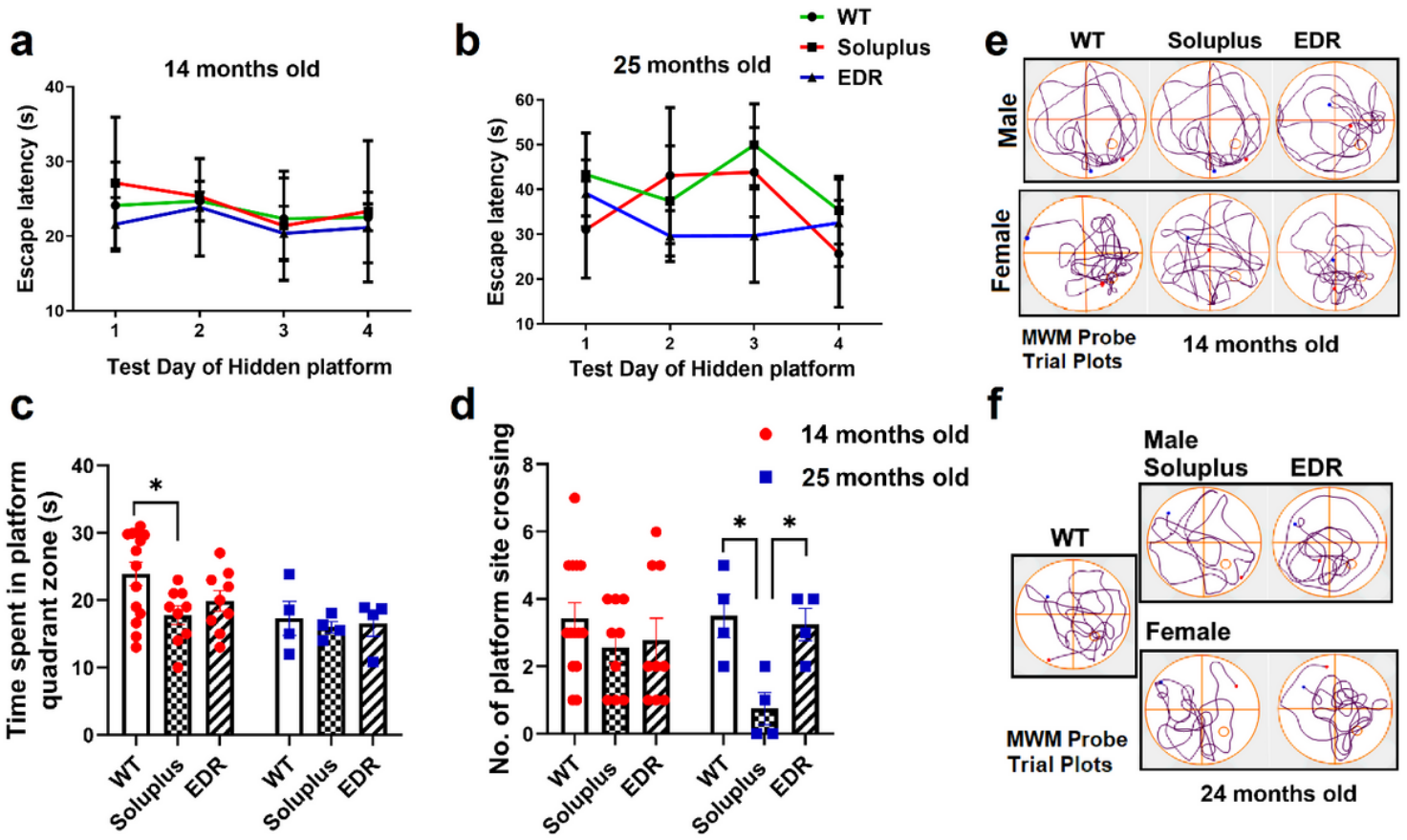

Figure 2

Acquisition learning and Probe trial in MWM test. (A, B) Hidden platform trials showing that both vehicle and EDR treated P301L mice in the 14 months old (A) and 25 months old group (B) learned to locate the hidden platform as shown by reduced escape latencies in the acquisition phase. Two-way ANOVA revealed no significant reduction of escape latency at day 4 compared to day 1 in all groups. In the 25 months old group (B), EDR treated mice showed more reduction in escape latency compared to WT and their control littermates, however, the difference was not significant. (C, D) (E, F) The track plots of the probe trial showing EDR increased the time spent in the target quadrant zone, where the platform was previously hidden, and the number of platform site crossing. All data are presented as mean ± SEM. *means significant difference, $p < 0.05$ (one-way ANOVA), n = 9-14/group at 14 months; n = 4/group at 25 months. Two-way ANOVA was used to analyze data for the escape latency during hidden platform trials (Panel A, B).

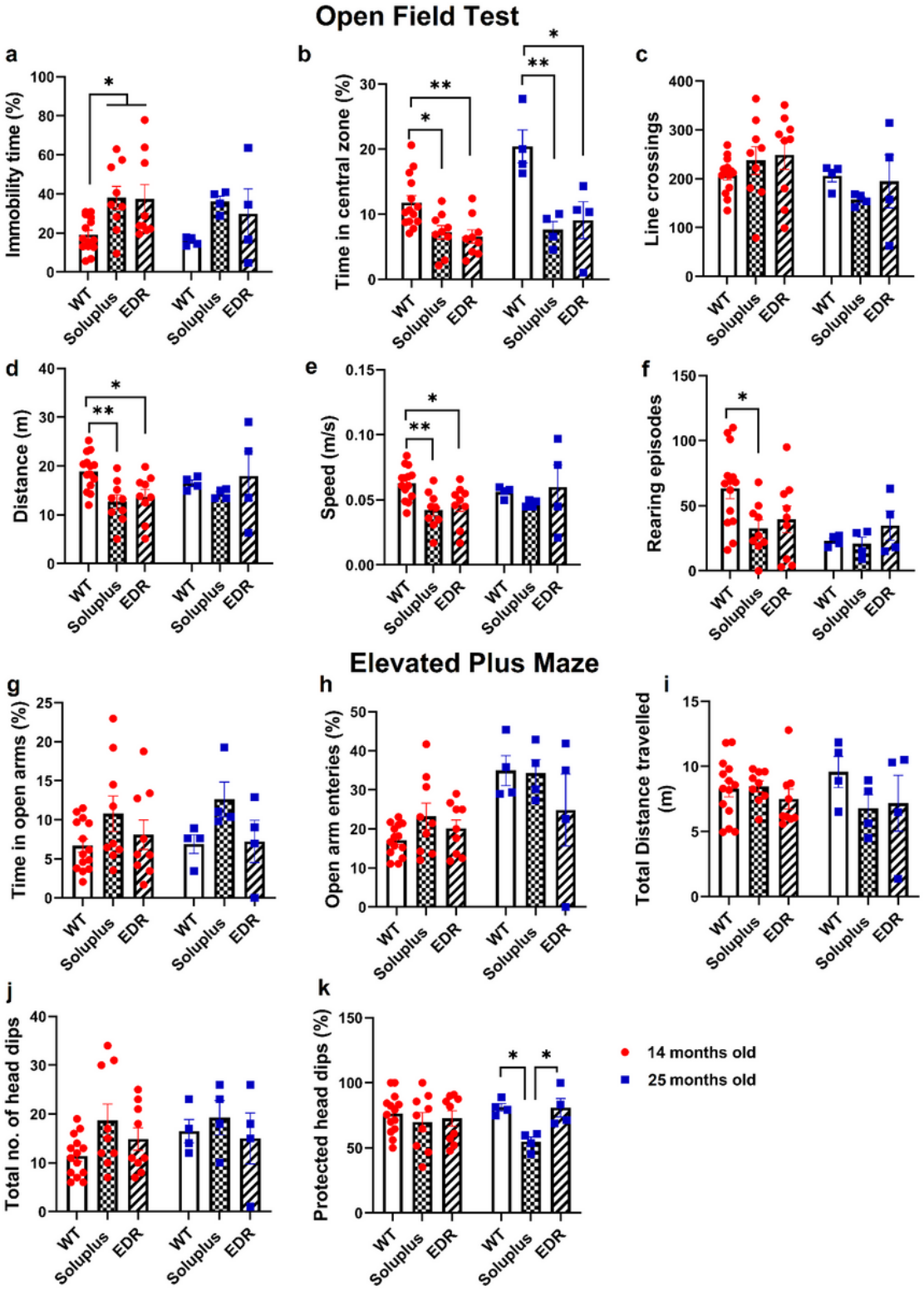

Figure 3

Open field and Elevated plus maze task. (A-F) Effect of EDR on anxiety and exploration of P301L mice in the open field task. Control P301L mice showed increased anxiety levels compared to WT littermates. EDR improved the exploratory behavior as evident by increasing the number of rearing episodes, and slightly improved the anxiety levels (A) Percentage of time spent immobile, (B) percentage of time spent in central (anxious) zone, (C) number of zone transitions or line crossing, (D) total distance travelled, (E)

average speed and (F) total number of rearing episodes. (G-K) Effect of EDR on P301L mice in elevated plus maze task. Control P301L mice showed increased exploration compared to WT littermates. EDR treatment slightly reversed that behavior as evident by reduced open arms visits and increased percentage of protected head dips. (G) Percentage of time spent in open arms, (H) percentage of open arm entries, (I), total distance travelled, (J) total number of head dips and (K) percentage of protected head dips to total head dips. Data represent mean ± SEM, one-way ANOVA, * significant difference $p < 0.05$ n = 9/group at 14 months, n = 4/group at 25 months.

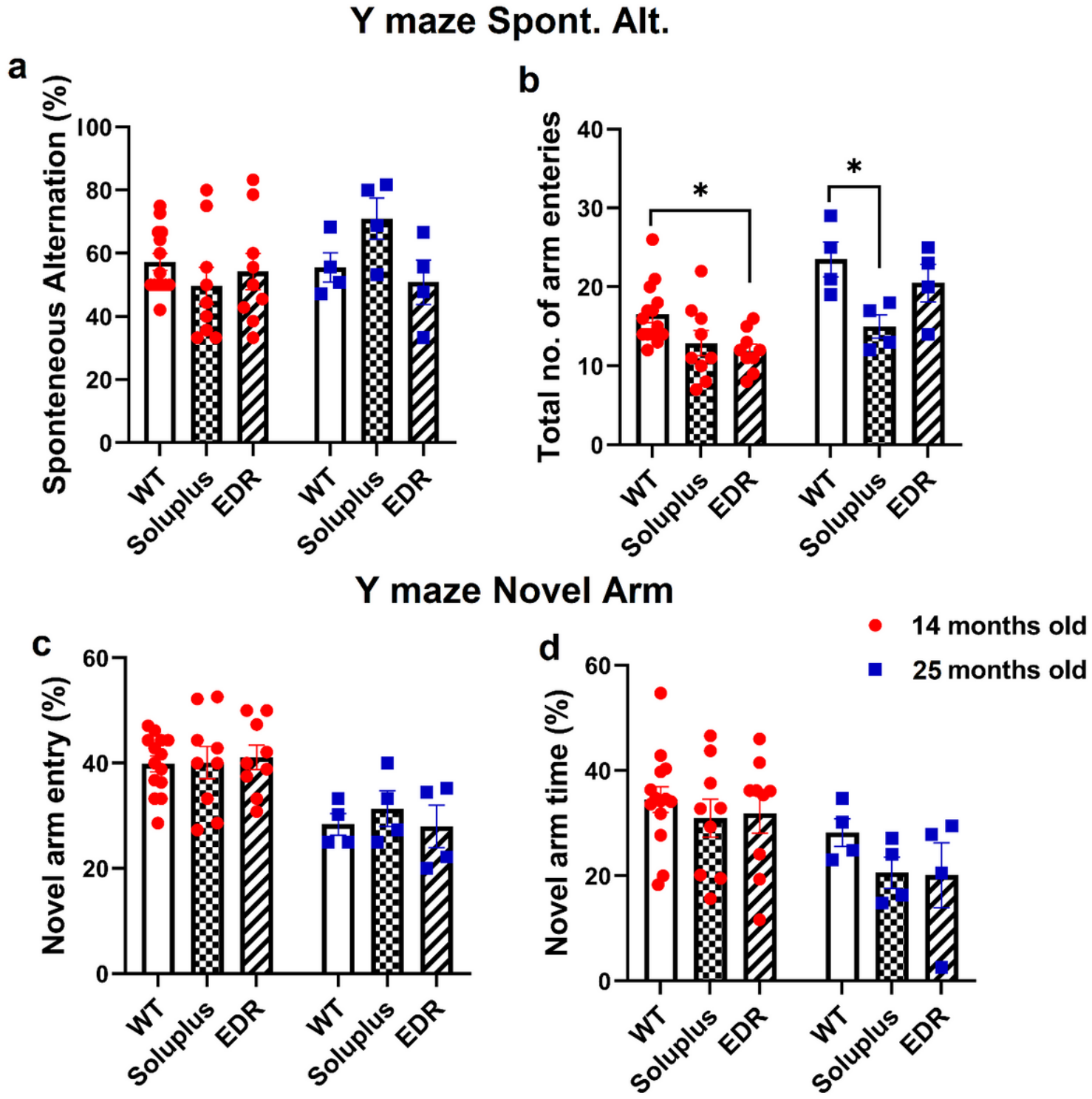

Figure 4

Y maze spontaneous alternation and Y maze novel arm tasks. (A-B) All groups showed the same percentage of alternation. But control P301L mice exhibited significantly lower number of total arm entries compared to WT mice in both age groups, which was slightly reversed by EDR in old age group. (C-D) No significant difference was observed between groups in Y maze novel arm test in 14 months old group. However, a trend towards impairment in the spatial working memory was noticed in P301L controls at 25 months, that was partially improved by EDR. Data represent mean ± SEM, one-way ANOVA, * significant difference $p < 0.05$ n = 9/group at 14 months old, n = 4/group at 25 months old.

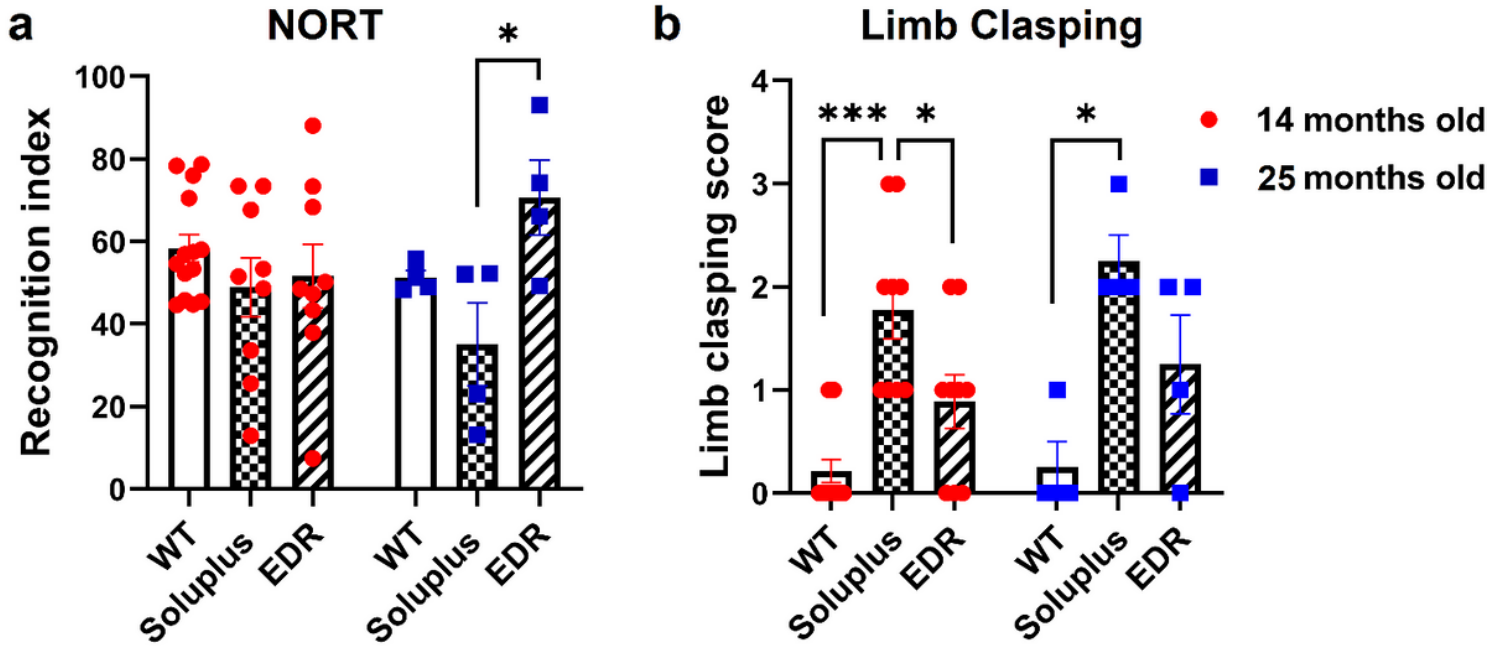

Figure 5

Effect of EDR on the recognition memory and motor function of P301L mice. (A) No significant difference was observed among groups at 14 months, indicating that working memory is not impaired at that age. At 25 months old, control P301L mice showed less preference to explore the novel object. EDR significantly increased the recognition memory in old P301L that performed superior to WT littermates. (B) P301L mice showed minor motor impairment as evident by increased score in the limb clasping test. EDR significantly reduced the limb clasping score at 14 months of age and inhibited motor function deterioration in older mice. Data represent mean ± SEM, one-way ANOVA, * significant difference $p < 0.05$ n = 9/group at14 months, n = 4/group at 25 months.

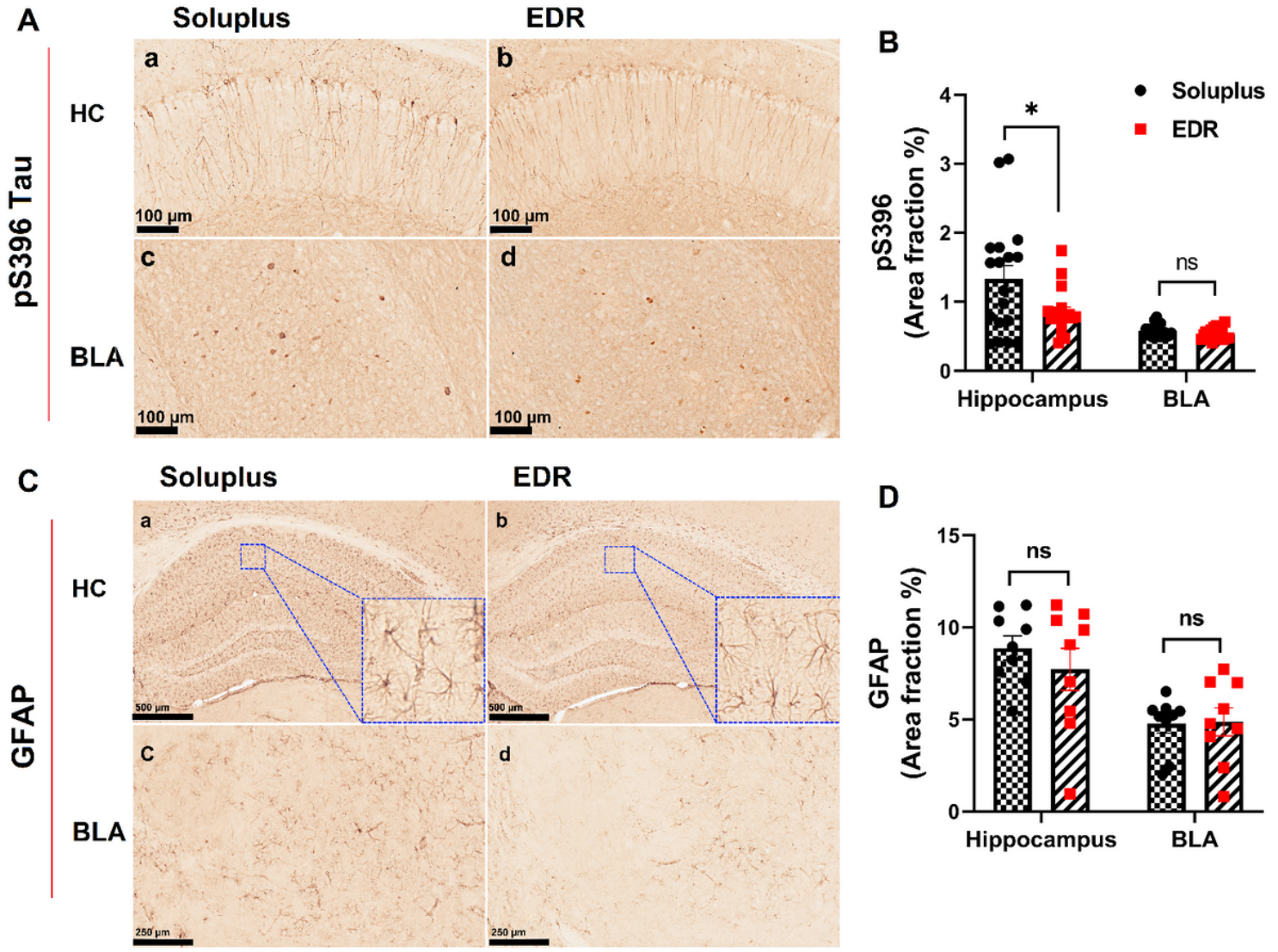

Figure 6

Immunostaining of coronal brain section from vehicle (Soluplus) and EDR-treated P301L mice (age = 14 months old) with anti-pS396 (A) and anti-GFAP (C). A (a and b) Images of hippocampus CA1 region and (c and d) of the amygdala region immune-stained with pS396 tau antibody (Scale bar = 100 µm). B is a quantitative graph of pS396 positive neurons in vehicle and EDR treated mice showing the EDR treatment significantly reduced the levels of pS396 significantly in the CA1 hippocampus regions and to some extent in the BLA. C (a and b) Images of the hippocampus and (c and d) of the amygdala region immune-stained with GFAP antibody (Scale bar = 500 µm for the upper panel and 40x magnification of the image is shown in inset, Scale bar = 250 µm for the lower panel). D is a quantitative graph of GFAP positive astrocytes showing non-significant difference in GFAP positive astrocytes in the hippocampus, and in the amygdala of control and EDR treated animals. *p < 0.05 vs. Soluplus treated mice (unpaired two-tailed t test). Two sections were analyzed from each group for phosphorylated Tau (n = 2/animal). Results are presented as means ± SEM.

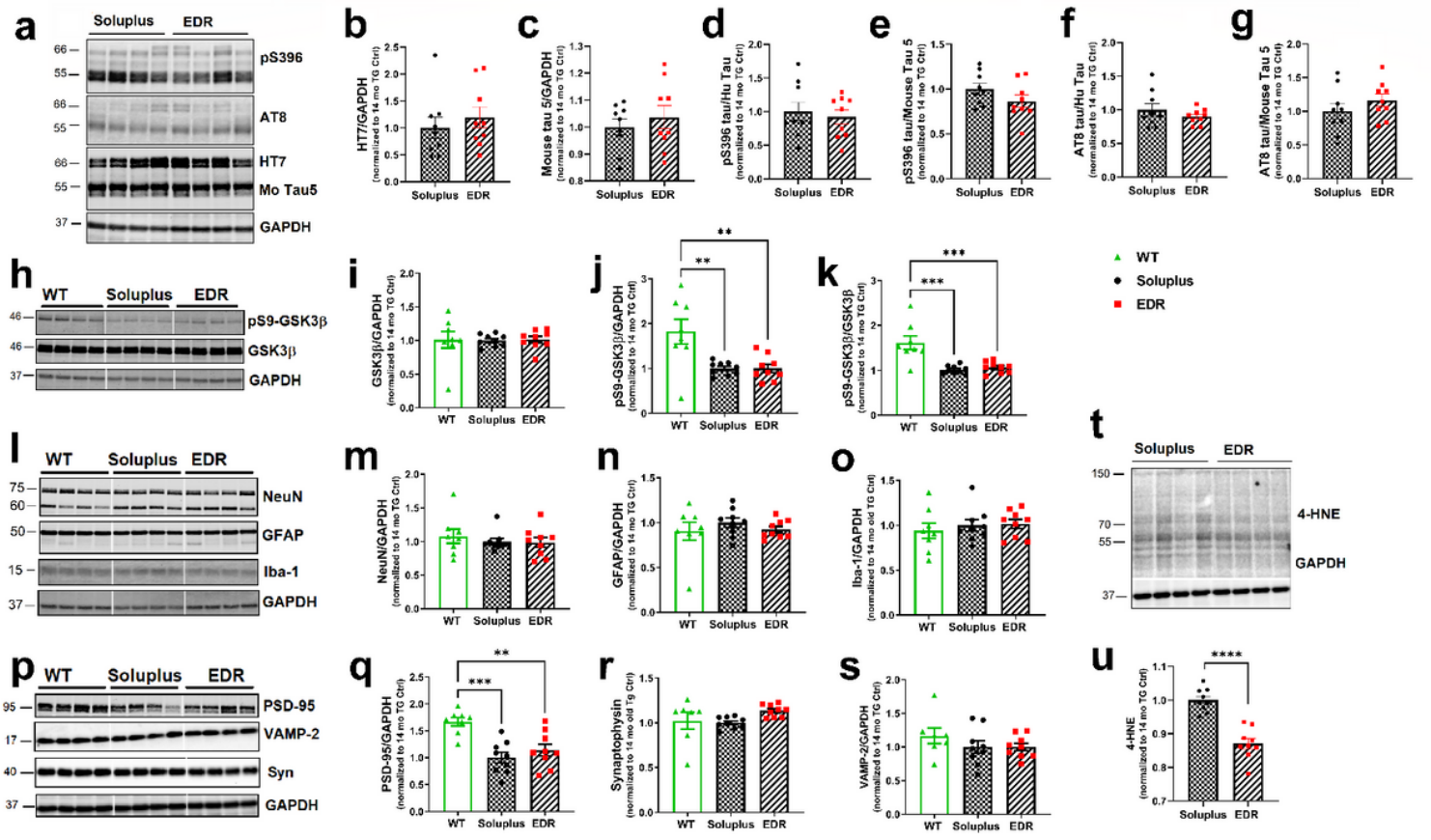

Figure 7

Western blot and their quantitative analysis of total brain homogenates of P301L mice at 14 months old probed with antibodies against (a-g) total and phosphorylated tau residues, (h-k) total GSK-3β, its phosphorylated form pS9, (l-o) NeuN, GFAP and Iba-1 (p-s) synapse-associated proteins including synaptophysin, PSD-95 and VAMP-2 and (t-u) 4-HNE modified protein levels. GAPDH was used as a loading control. Results showing the expressions levels of proteins normalized to vehicle control P301L mice (age 14 months old). *p < 0.05, **p < 0.01, *** p < 0.0001vs. Soluplus treated mice (unpaired two-tailed t test and one-way ANOVA). Results are presented as means ± SEM.

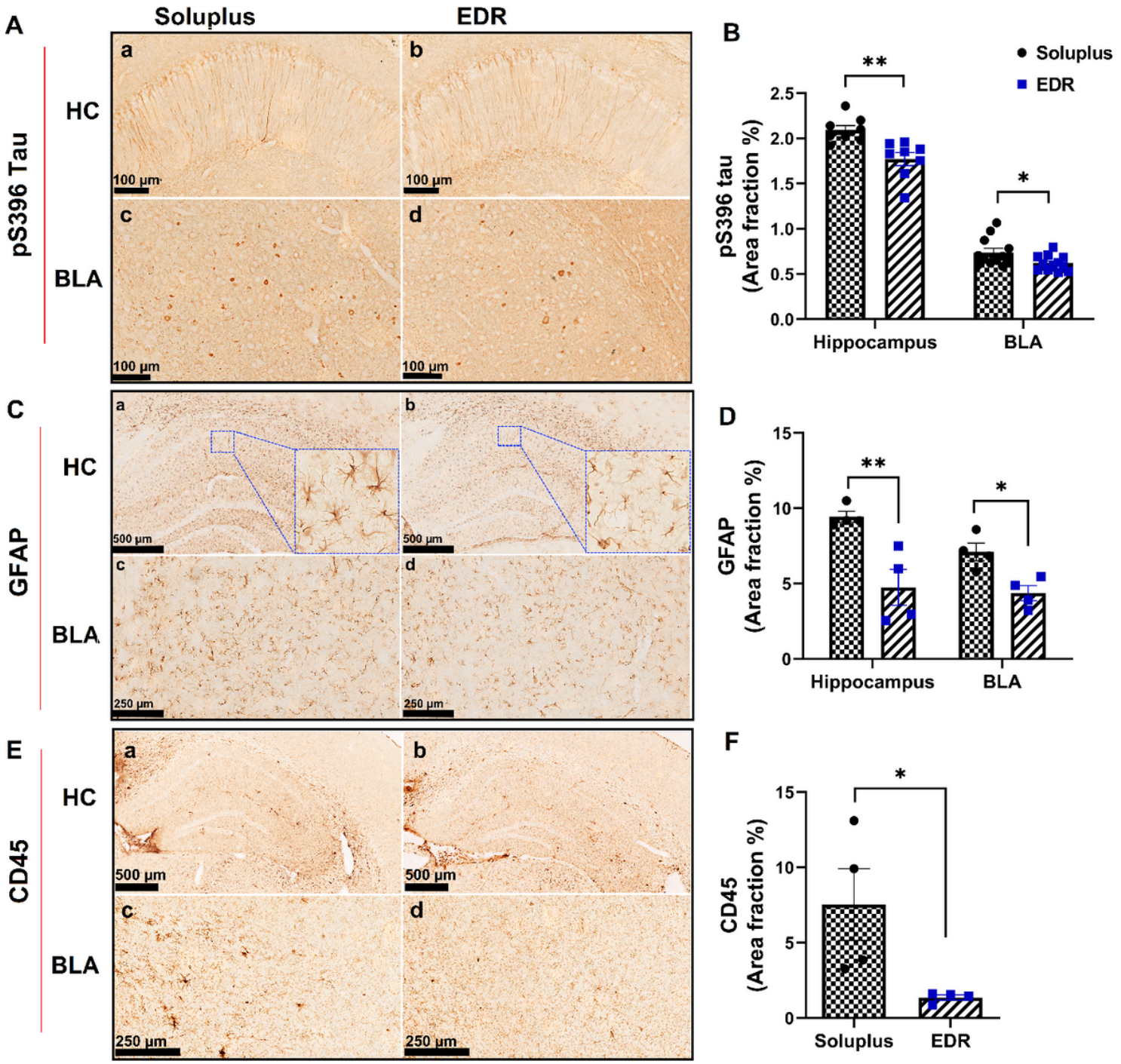

Figure 8

Immunostaining of coronal brain section from vehicle (Soluplus) and EDR-treated P301L mice (age = 25 months old) with anti-pS396 (A), anti-GFAP (C) and anti-CD45 (E). A (a and b) Images of CA1 region of the hippocampus (c and d) and amygdala region immune-stained with pS396 tau antibody (Scale bar = 100 µm). B is the % of area fraction quantification of pS396 positive neurons in vehicle and EDR treated mice showing the EDR treatment significantly reduced the levels of pS396 in the CA1 hippocampus regions and BLA. C (a and b) Images of the hippocampus and (c and d) of the amygdala region immune-stained with GFAP antibody (Scale bar = 500 µm for the upper panel and 40x magnification of the image

is shown in inset, Scale bar = 250 µm for the lower panel; c and d). D is a quantitative graph of GFAP represented as % of area fraction showing significant reduction in GFAP positive astrocytes in both the hippocampus and amygdala of EDR treated mice compared to vehicle controls. E (a and b) Images of the hippocampus and (c and d) amygdala region immune-stained with CD45 antibody (Scale bar = 500 µm; upper panel, and Scale bar = 250 µm for lower panel). F is a representative quantification (% of area fraction) of CD45 positive microglia showing that EDR treatment significantly reduced the overall expression levels of CD45 in the brain. Results are presented as mean ± SEM.*p < 0.05 vs. Soluplus treated mice (unpaired two-tailed t test).

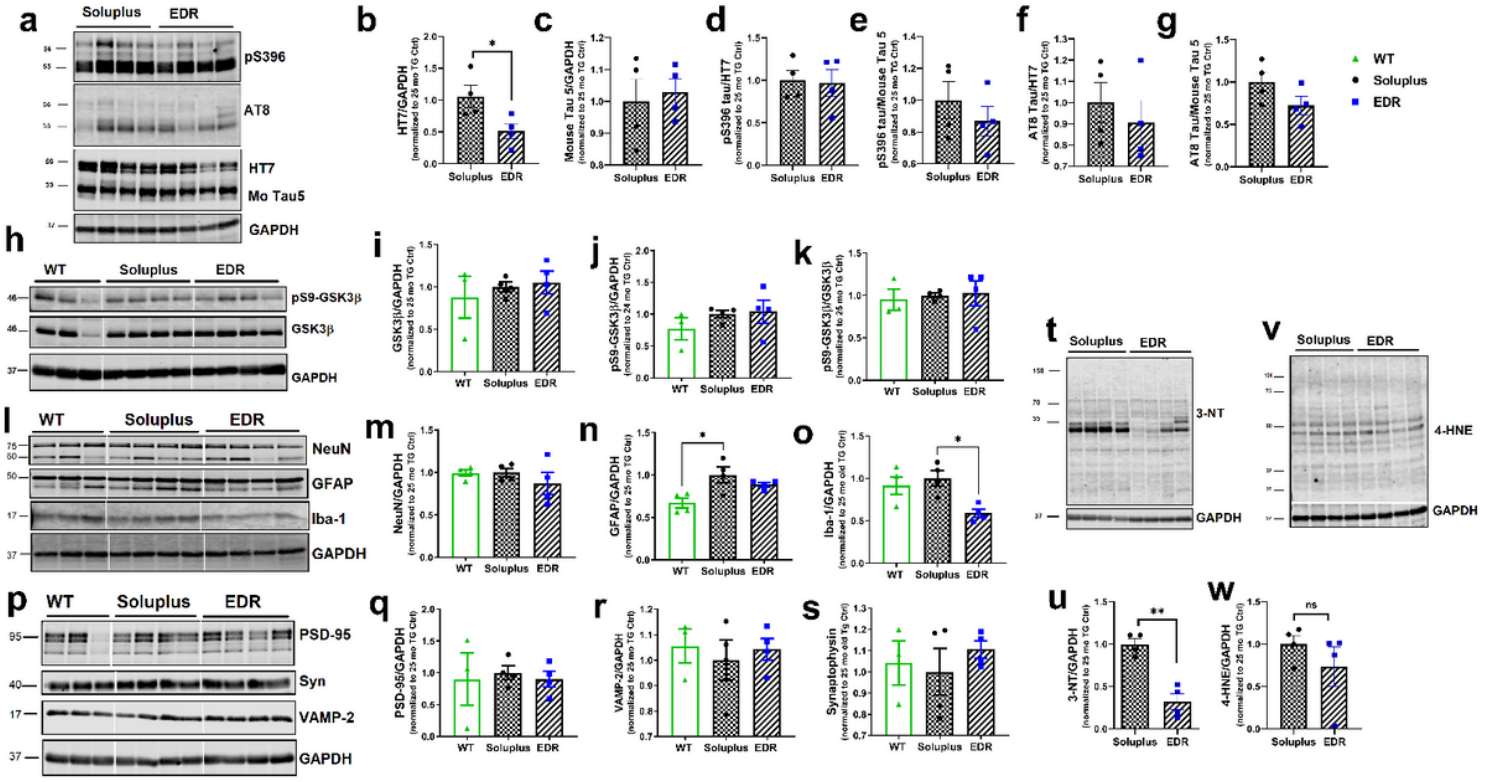

Figure 9

Western blot and their quantitative analysis of total brain homogenates of P301L mice at 25 months old probed with antibodies against (a-g) total and phosphorylated tau residues, (h-k) total GSK-3β, its phosphorylated form pS9 (l-o) NeuN, GFAP and Iba-1 (p-s) synapse-associated proteins including synaptophysin, PSD-95 and VAMP-2, (t-u) 3-NT modified protein levels and (v-w) 4-HNE modified proteins levels. GAPDH was used as a loading control. Results showing the expressions levels of proteins normalized to vehicle control P301L mice (age 25 months old). *p < 0.05, **p < 0.01 vs. Soluplus treated mice (unpaired two-tailed t test and one-way ANOVA). Results are presented as means ± SEM.

Supplementary Files

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- Supplementarymaterial.docx